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Caleb Stubbs

Pepperdine University

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Summer Undergraduate Research
in Biology



VEGFR-2 and VEGFR-3 Expression as a Function of Jagged-1 Over-Expression in HUVEC Cells

Caleb Stubbs

Mentor: Dr. Thomas Vandergon
Pepperdine University Malibu, California 90263

UNIVERSITY of NORTH CAROLINA
PEMBROKE



Abstract

Angiogenesis is the sprouting of new blood vessels from pre-existing vessels initiated from vascular endothelial cells in response to exogenous chemical signals. Principle signals are vascular endothelial growth factors (VEGF) that direct cell growth and differentiation by binding to endothelial cell surface VEGF receptors (VEGFR). In Human Umbilical Vascular Endothelial Cells (HUVEC) initial sprouting cells or “tip cells” send signals to neighboring cells “stalk cells” responsible for sprout elongation. Multiple VEGF signals and receptors are known, but the main angiogenic signals are from VEGF-A through VEGFR-2 and VEGFR-3. Regulation of VEGF receptor expression can be influenced by the Notch signaling pathway that involves Delta Like Ligand 4 (Dll4) interaction with and its receptor, Notch-1, but also may be influenced by backtalk through a second Notch ligand, Jagged-1. We examined how Jagged-1 overexpression may affect VEGFR-2 and VEGFR-3 gene expression in HUVECs cells that are in “Tip Cell” mode vs. “Stalk Cell” mode. We found that overexpressing Jagged-1 in HUVECs that are not communicating through Notch increases VEGFR-2 and VEGFR-3 receptor expression but decrease these levels when cells can “talk” through Notch.

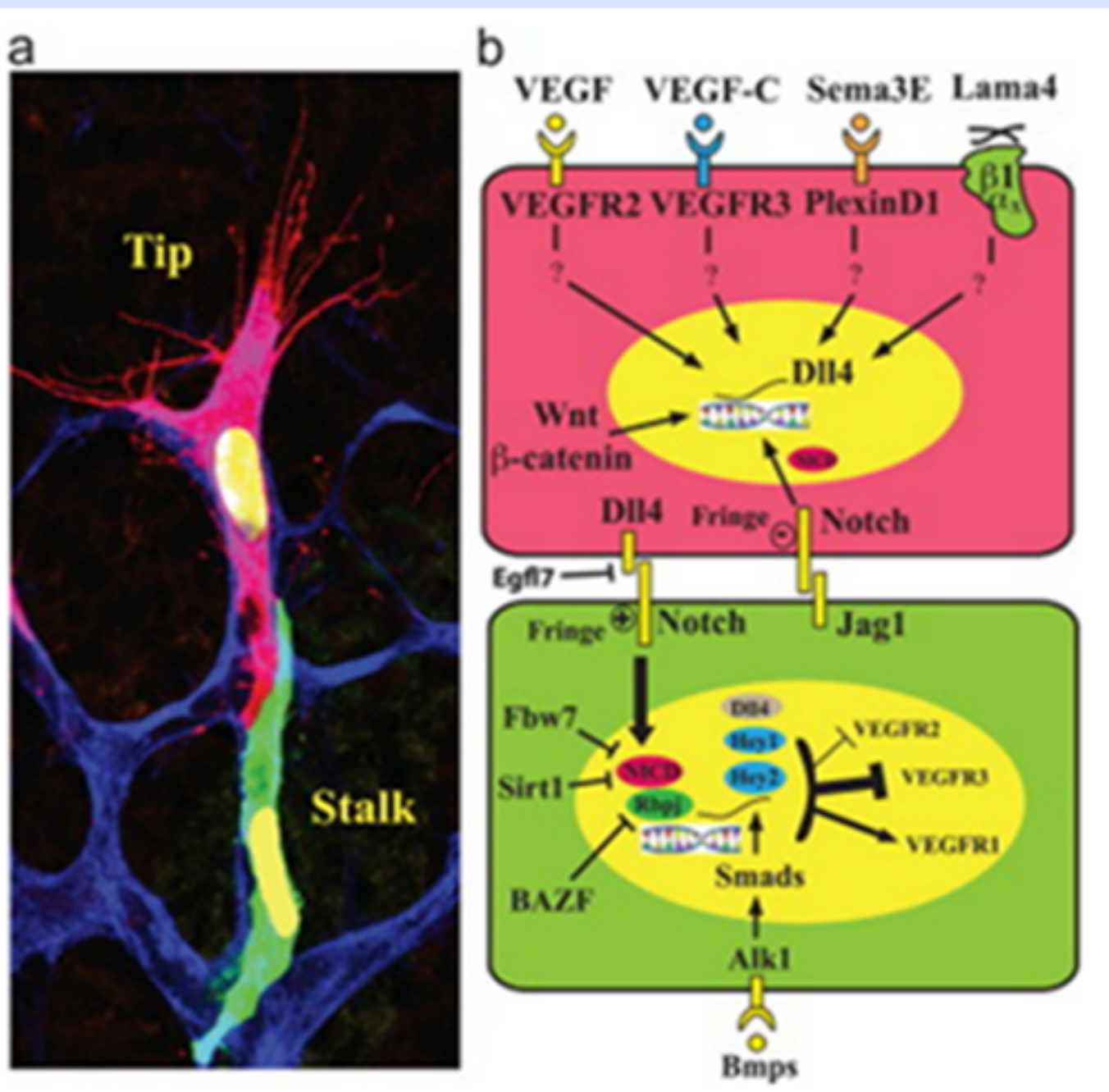
Introduction

Angiogenesis is initiated by the binding of specific glycoproteins called Vascular Endothelial Growth Factors (VEGF) to transmembrane receptors (VEGFR) on the cell surface. There are 5 VEGF molecules: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF (placental growth factor) (1, 8). VEGF-A is known to be heavily involved in vascular vessel development and VEGF-C is more directed towards lymph vessel development or lymphangiogenesis. The VEGF receptors are transmembrane tyrosine kinases which once bound to a ligand either hetero- or homo-dimerize resulting in self and cross phosphorylation of tyrosine residues on their intracellular domains, causing a cascade of downstream pathways to be activated (1).

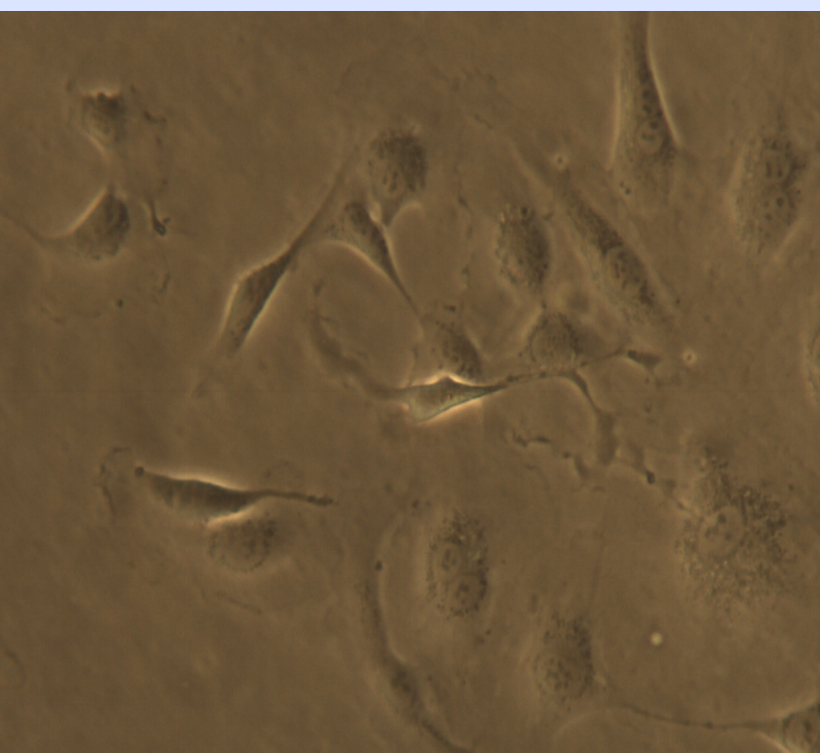
In Human Umbilical Vein Endothelial Cells (HUVECs) initiating angiogenesis, VEGF-A typically binds to VEGFR-2 and VEGF-C binds to VEGFR-3 but there has been studies shown that they can cross talk (1). VEGF A signaling in HUVECs cells causes initial sprouting of some cells known as “tip cells” which talk through the Notch signal pathway to adjacent cells called “stalk cells”. When VEGFR-2, the main receptor involved in angiogenesis, is phosphorylated the Notch-1 ligand known as “Delta Like Ligand 4” (Dll4) is up regulated within the cell. When Dll4 binds to the Notch-1 receptor in a neighboring cell, the Notch Intracellular domain (NICD) is cleaved by proteases and gamma-secretases. NICD is then transported to the nucleus where it forms complexes with transcription repressors to become transcription activators wherein it is capable of binding to DNA and allows transcription of certain genes to occur (4, 5). Genes such as *Hey1*, *Hey2*, and *Narp* that are up regulated due to Notch signaling play roles in cell proliferation and differentiation. The expression of VEGF receptors also differ depending on whether Dll4/Notch signaling is occurring. In tip cells where signaling for sprouting is important, VEGF-A is up regulated along with the Notch ligand Dll4 to help signal other cells not to respond to the VEGF-A (5, 6). In stalk cells we see an increase in Notch-1, and VEGFR-3, while decreases in Dll4 and VEGFR-2 (6, 7).

Interestingly, another ligand to Notch-1 known as Jagged-1, has been found to be up regulated when Notch signaling is occurring within vascular endothelial cells. It has been supposed that Jagged-1 acts as a competitive ligand to Dll4 and may alter Dll4 signaling between tips cells and stalk cells. Evidence on whether or not Jagged-1 plays a role in Notch signaling has yet to be shown. The objective of this project was to identify a difference in expressing of VEGFR-2 and VEGFR-3 between tip and stalk cells by over-expression of Jagged-1. Our goal was to see if there are any effects of Jagged-1 overexpression that are comparable when Dll4/ Notch signaling is occurring or not.

Diagram of VEGF and NOTCH Signaling in Tip vs. Stalk Cell Formation in Angiogenesis.



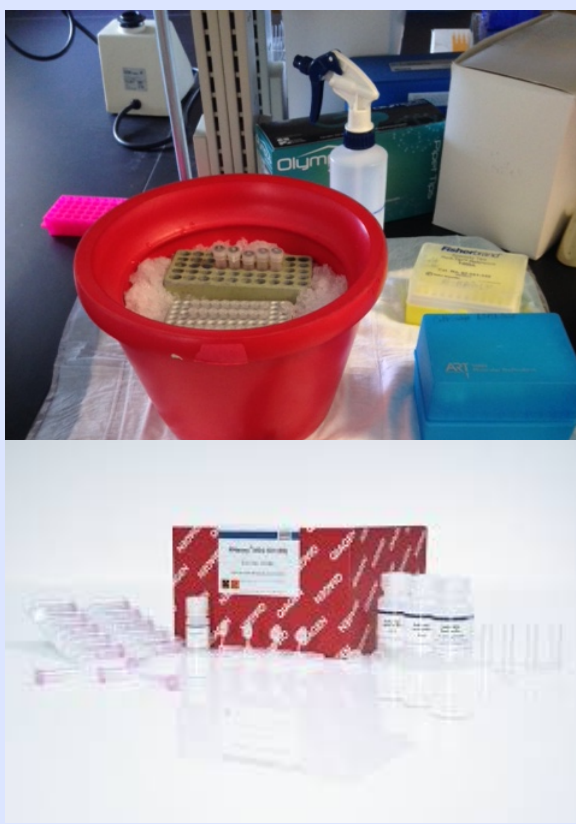
Methods



M1. HUVECs culture plated in (-)VEGF EGM2-MV medium (Lonza) for 24 hr growth period. “Tip Cell-Like” plates (10cm) received 3.5×10^5 cells while “Stalk Cell-Like” plates (60mm) received 5×10^5 .



M2. HUVECs cultures were transfected with Jagged-1 construct or GFP control construct in both “Tip Cell-Like” plates (10cm) and “Stalk Cell-Like” plates (60mm). 2.2 µg/ 60 mm plate and 4.4 µg/10 cm plate. Lipofectamine LTX with Plus Reagent (Invitrogen, Inc.) at 1.0 µl per µg of DNA was used for each transfection assay.

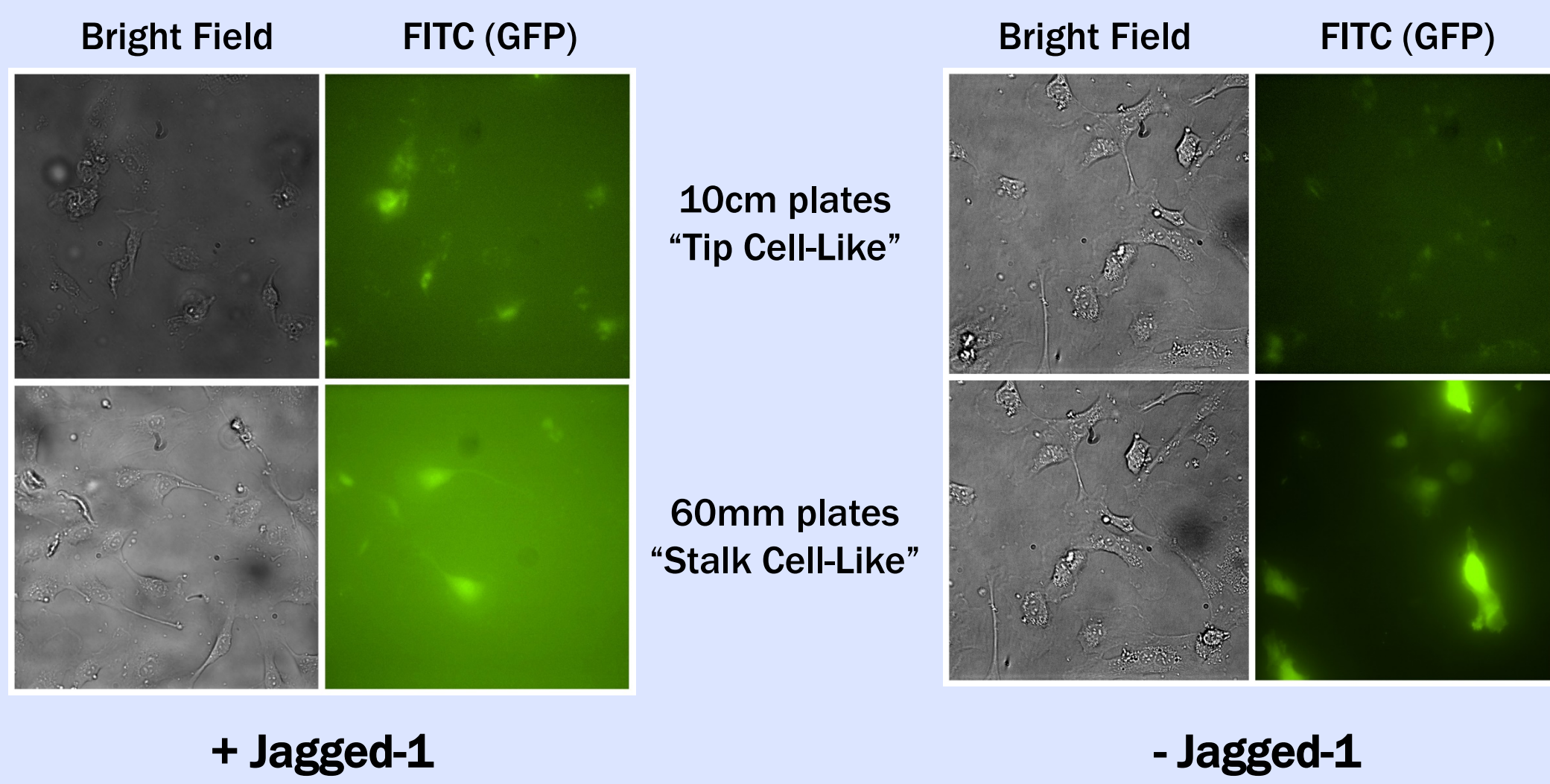


M3. 48 hr post-transfection HUVECs cell cultures were harvested and the total RNA was extracted from the collected cells using the Qiagen Rneasy Mini Kit following the manufacturer's instructions. RNA concentrations were quantified on a Nanodrop 1000 spectrophotometer at 260 nm.



M4. Total RNA from the transfected HUVECs cultures were analyzed for VEGFR-2 and VEGFR-3 expression using Taqman® qRT-PCR protocol and the Quanta Biosystems qScript One-Step Fast Low-Rox qRT-PCR kit. 6 ng of total RNA were used in each reaction. β-actin was amplified as an internal control.

Results



	60 mm (Stalk Cell-Like)	10 cm (Tip Cell-Like)
+ Jagged Construct	33.4%	80.0%
+ Control GFP Construct	19.9%	55.3%

Figure 1. Bright field and FITC filter pictures for HUVECs transfected with either Jagged-1 construct (+Jagged-1) or control GFP construct (-Jagged-1). Images are shown for 10 cm plates in “Tip Cell-Like” growth as well as for 60 mm plates in “Stalk Cell-Like” growth. Jagged-1 constructs were co-transfected with the GFP control plasmid at 10% control concentration. Transfection efficiency as a percent of bright field cells was determined at 48 hour post-transfection. Transfection efficiency for each group is shown in the table to the right.

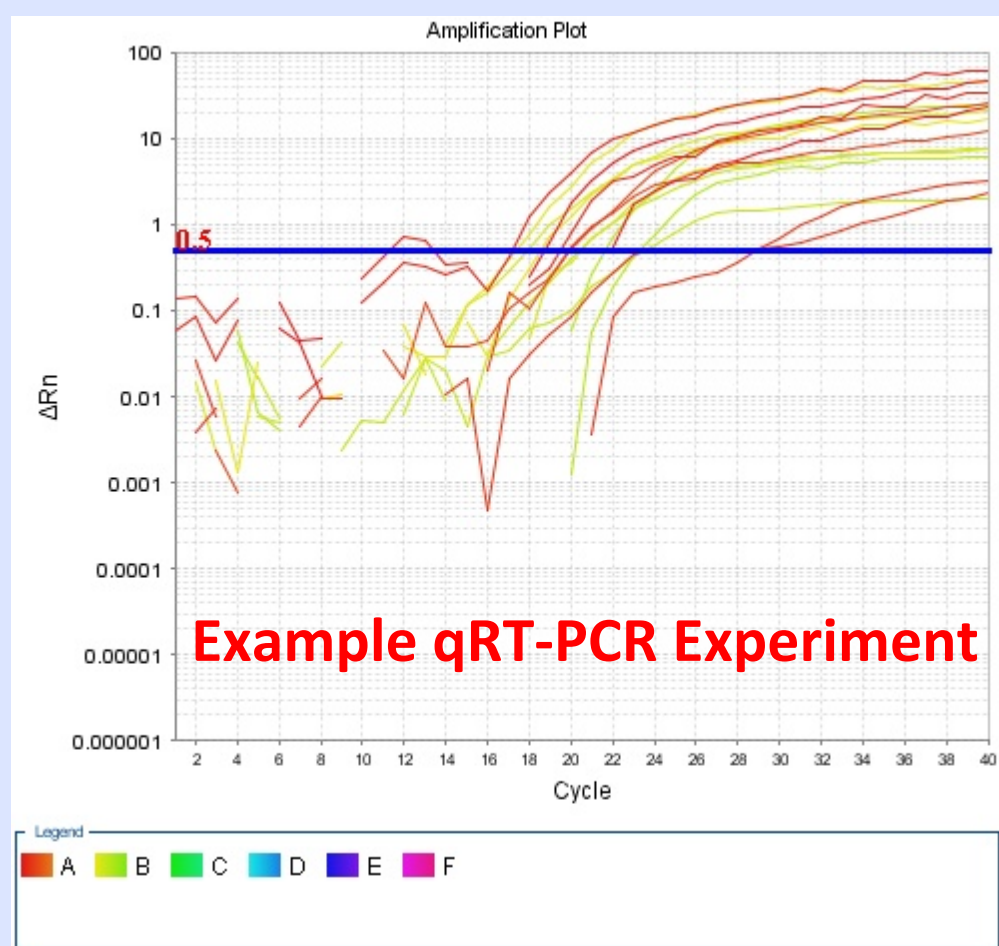
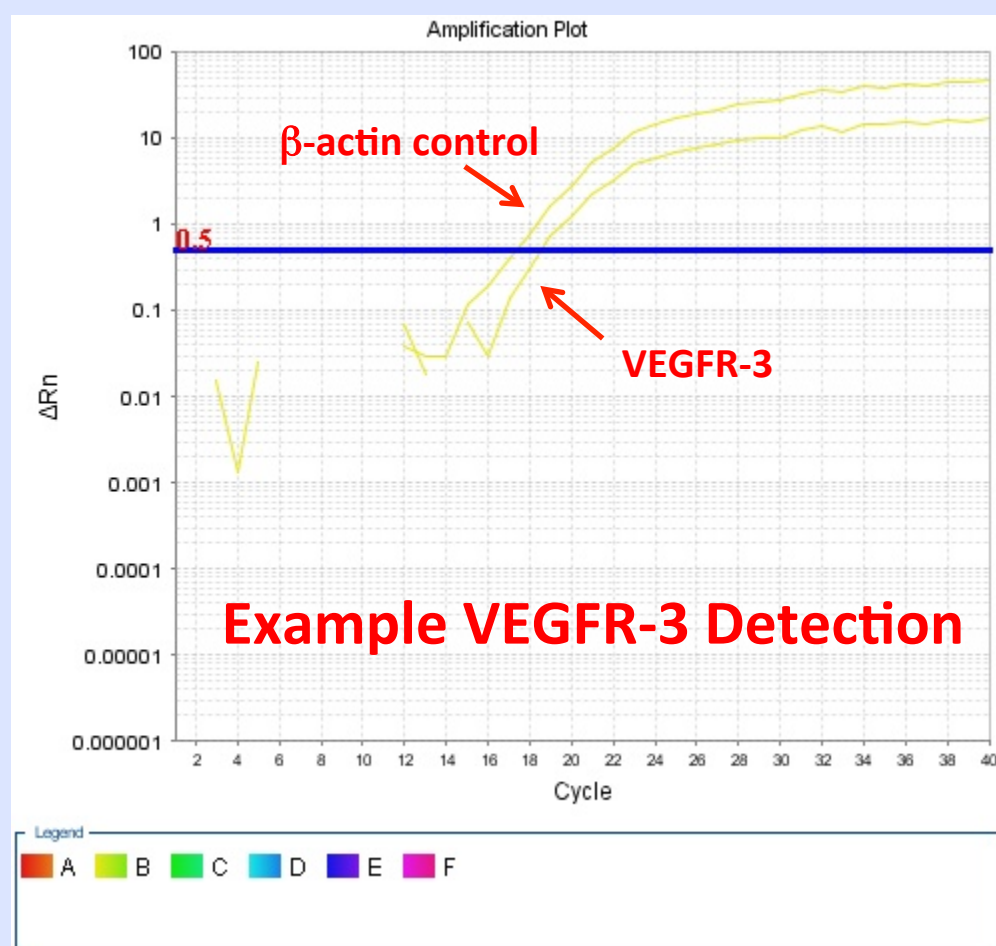
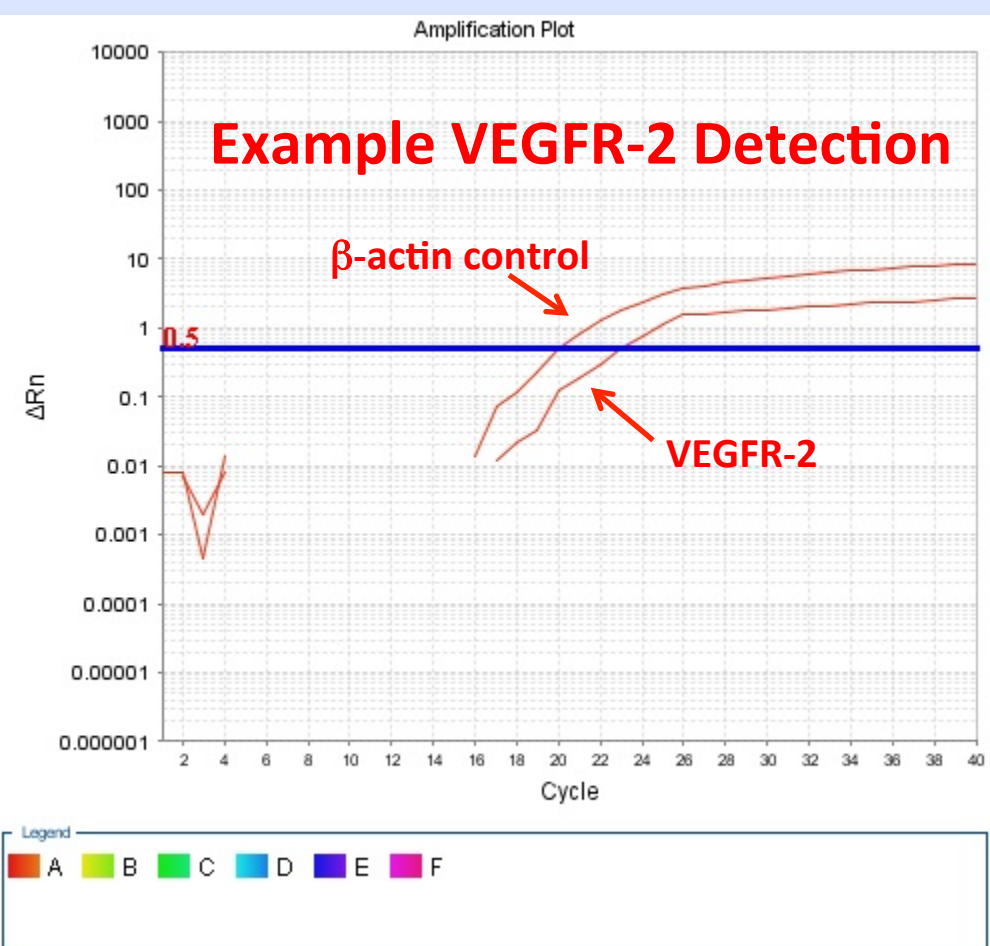


Figure 2. Plots showing ΔR_n , which is a function of target fluorescence, as a function of cycle number for a full experiment (left), a sample VEGFR-2 experiment (middle) and a sample VEGFR-3 experiment (right). Two replicates for four independent experiments were done for each plate set. VEGFR-2 and VEGFR-3 primers were used to amplify DNA which was detected by a specific Taqman® probe for each. β-actin amplification and detection with a Taqman® probe was used as an endogenous control.



Results Summary

- There were differences in growth and appearances of cells in the +Jagged-1 transfections compared the control transfections. The worst growth was in the 10 cm “Tip Cell-Like” + Jagged-1 plates.
- The percent transfections in both Jagged-1 and control constructs were within or better than published expectations, but were consistently higher in the 10 cm plates compared to the 60 mm plates.
- Both VEGFR-2 and VEGFR-3 expression increased when HUVECs were in “Tip Cell-Like” growth relative to “Stalk Cell-Like” growth.
- In “Stalk Cell-Like” growth, overexpression of Jagged-1 results in decreased expression of both VEGFR-2 and VEGFR-3.

Conclusions

- In HUVEC cells, “tip” and “stalk” cell signaling competition occurs. Thus the fates of tip and stalk cells change depending on the balance of VEGF and Notch signaling (6).
- When Jag-1 is over-expressed, in “Stalk” cell mode, VEGFR-2 and VEGFR-3 expression is decreased compared to the levels in “Tip” cell mode where the receptor expression levels increase.
- In “Tip” cells that are not in contact with other cells, the Jag-1 response most likely is due to Jag-1 intracellular domain self signaling.
- In “Stalk” cells the Jag-1 response is most likely due to communication cell to cell through the binding to Notch.

Sequence Name	Sequence (5'-3')
Stubbs VEGFR2 Forward	AGCGATGGCTCTTCTGTAA
Stubbs VEGFR2 Reverse	ACACGACTCATGTTGGTCA
Stubbs VEGFR2 Probe	TGATCGGAATGACACGTGA
Stubbs VEGFR3 Forward	GAGACAAGGACAGCGAGGAC
Stubbs VEGFR3 Reverse	TCACGAACACGTAGGAGCTG
Stubbs VEGFR3 Probe	GTACATGCCAAGCAGACACAGG
Stubbs β-actin Forward	GGACTTCGAGCAGAGATGG
Stubbs β-actin Reverse	AGCACTGTGTTGGGTCACG
Stubbs β-actin Probe	CTTCCAGCCTTCTCTCT

Figure 3. Table of VEGFR-2, VEGFR-3, β-actin primer and probe sequences.

Acknowledgments

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Figure 3. Graph showing the $\Delta\Delta C_t$ values for VEGFR-2 and VEGFR-3 gene expression based on qRT-PCR results when normalized to and then subtracted from β-actin values. Significant differences were observed between the VEGFR-2 groups indicated by the letter ‘a’.

